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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 5/00, 5/06	A2	(11) International Publication Number: WO 98/04681 (43) International Publication Date: 5 February 1998 (05.02.98)																					
<p>(21) International Application Number: PCT/US97/13140</p> <p>(22) International Filing Date: 25 July 1997 (25.07.97)</p> <p>(30) Priority Data:</p> <table border="0"><tr><td>60/022,801</td><td>25 July 1996 (25.07.96)</td><td>US</td></tr><tr><td>60/022,810</td><td>25 July 1996 (25.07.96)</td><td>US</td></tr><tr><td>60/022,711</td><td>26 July 1996 (26.07.96)</td><td>US</td></tr></table> <p>(60) Parent Applications or Grants</p> <p>(63) Related by Continuation</p> <table border="0"><tr><td>US</td><td>60/022,801 (CIP)</td></tr><tr><td>Filed on</td><td>25 July 1996 (25.07.96)</td></tr><tr><td>US</td><td>60/022,810 (CIP)</td></tr><tr><td>Filed on</td><td>25 July 1996 (25.07.96)</td></tr><tr><td>US</td><td>60/022,711 (CIP)</td></tr><tr><td>Filed on</td><td>26 July 1996 (26.07.96)</td></tr></table> <p>(71) Applicant (for all designated States except US): GENZYME CORPORATION [US/US]; One Mountain Road, Framingham, MA 01701 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): MCPHERSON, John, M. [US/US]; 378 Elm Street, Hopkinton, MA 01748 (US).</p>		60/022,801	25 July 1996 (25.07.96)	US	60/022,810	25 July 1996 (25.07.96)	US	60/022,711	26 July 1996 (26.07.96)	US	US	60/022,801 (CIP)	Filed on	25 July 1996 (25.07.96)	US	60/022,810 (CIP)	Filed on	25 July 1996 (25.07.96)	US	60/022,711 (CIP)	Filed on	26 July 1996 (26.07.96)	<p>YAEGER, Peter, C. [US/US]; 19 Robin Hood Road, Natick, MA 01760 (US). BROWN, Marie, E. [NL/US]; 21 Mosman Street, West Newton, MA 02165 (US). HANLON, James, G. [US/US]; 105 High Street, Ashland, MA 01721 (US). BINETTE, François [CA/US]; 40 Crescent, Belmont, MA 02178 (US).</p> <p>(74) Agents: GOSZ, William, G. et al.; Genzyme Corporation, One Mountain Road, Framingham, MA 01701 (US).</p> <p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
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<p>(54) Title: CHONDROCYTE MEDIA FORMULATIONS AND CULTURE PROCEDURES</p> <p>(57) Abstract</p> <p>One object of the present invention is based upon the development and use of a serum-free defined cell culture medium comprising a supplement mixture, a component mixture, a vitamin mixture, an inorganic salt mixture and amino acid mixture that avoids the problems inherent in the use of serum. In particular, the defined medium is useful in culturing fibroblasts, especially chondrocytes. Another object of the present invention is to claim a method of enhancing the differentiation of chondrocytes and enhancing the synthesis of a cartilage specific matrix using tumor growth factor beta (TGF-β). Another object of the present invention is to claim a method of enhancing the differentiation of chondrocytes using the combination of TGF-β and IGF.</p>																							

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CHONDROCYTE MEDIA FORMULATIONS AND CULTURE PROCEDURES

Cross-Reference to Related Applications

This application is related to and claims the benefit of provisional application serial number 60/022,810, filed July 25, 1996, provisional application serial number 60/022,711, filed July 26, 1996, and provisional application serial number 60/022,801, filed July 25, 1996.

Background of the Invention

Initially, the successful culture of mammalian cells *in vitro* required supplementation of growth medium with serum which provides hormones and growth factors necessary for cell attachment and proliferation. Although serum is still widely used for mammalian cell culture, there are several problems associated with its use (Freshney, Serum-free media. In *Culture of Animal Cells*, John Wiley & Sons, New York, 91-99, 1994): 1) serum contains many unidentified or non-quantified components and therefore is not "defined"; 2) the composition of serum varies from lot to lot, making standardization difficult for experimentation or other uses of cell culture; 3) because many of these components affect cell attachment, proliferation, and differentiation, controlling these parameters, or studying the specific requirements of cells with respect to these parameters, is precluded by the use of serum; 4) some components of serum are inhibitory to the proliferation of specific cell types and to some degree may counteract its proliferative effect, resulting in sub-optimal growth; and 5) serum may contain viruses which may affect the outcome of experiments or provide a potential health hazard if the cultured cells are intended for implantation in humans.

Primarily for research purposes, there has been some effort to develop biochemically defined media (DM). DM generally includes nutrients, growth factors, hormones, attachment factors, and lipids. The precise composition must be tailored for the specific cell type for which the DM is designed. Successful growth in DM of some cell types, including fibroblasts, keratinocytes, and

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epithelial cells has been achieved (reviewed by Freshney, 1994). However, attachment and proliferation of cells in DM is often not optimal.

One potential application of defined medium is the expansion of chondrocytes released from adult human articular cartilage for treatment of cartilage defects with autologous chondrocyte transplantation (Brittberg *et al*, New England Journal of Medicine, 331:889-895, 1994). Because this procedure involves the implantation of expanded chondrocytes into a patient, it may be desirable to avoid the use of serum or other undefined components during culture of the chondrocytes. For this application, the DM would need to sustain proliferation of adult human articular chondrocytes seeded at low density until confluent cultures are attained.

Several investigators have reported proliferation of high density non-articular chondrocytes in DM (Kato *et al*, Exp. Cell Res., 125:167-174, 1980; Madsen *et al*, Nature, 304:545-547, 1983; Quarto *et al*, Bone, 17:588, 1995). Others have reported proliferation of rabbit and human articular chondrocytes in DM (Boumedienne *et al*, Cell Prolif., 28:221-234, 1995; Schwartz, J. Clin. Chem. Clin. Biochem. 24:930-933, 1986). However, in these cases, chondrocytes were tested for growth in DM at high density ($\geq 20,000$ cells/cm²). Jennings and Ham (Cell Biology International Reports, 7:149-159, 1983) developed a serum-free medium for proliferation of chondrocytes isolated from costal cartilage of prepubertal humans and seeded at low density. That medium required the use of polylysine-coated plates and included a liposome mixture for which the authors state that there are "inherent limitations in the degree of chemical definition".

Attempts to culture articular chondrocytes at sub-confluent densities in DM have not been successful. Adolphe *et al* (Exp. Cell Res., 155:527-536, 1984) have developed a DM (Ham's F12 supplemented with insulin, transferrin, selenite, fibronectin, bovine serum albumin, brain growth factor, fibroblast growth factor, hydrocortisone, and multiplication stimulating activity --now known as Insulin-like growth factor II) which supports proliferation of rabbit articular chondrocytes. However, they report that serum-containing medium is necessary for the initial attachment of cells to the tissue culture vessel after seeding.

It has been reported that chondrocytes produce and secrete factors that promote their own attachment and proliferation (Shen *et al*, Endocrinology, 116:920-925, 1985). Examples include basic fibroblast growth factor (Hill *et al*, Growth Factors, 6:277-294, 1992), insulin-like growth factors (Froger-Gaillard *et al*, Endocrinology 124:2365-2372, 1989), transforming growth factor- β (Villiger, P.M. *et al.*, J. Immunol., 151:3337-3344, 1993), vitronectin, and possibly some unidentified factors that promote their attachment and proliferation. Because articular cartilage is a non-vascularized tissue, and the chondrocytes embedded in cartilage have limited access to systemic growth factors, autocrine stimulation may play an important role in the maintenance and proliferative capacity of these cells. To our knowledge, autocrine stimulation of chondrocytes has not been utilized for the purpose of enhancing the proliferation of human articular chondrocytes in DM.

During expansion in monolayer *in vitro*, articular chondrocytes de-differentiate, decreasing synthesis of matrix molecules normally produced by differentiated articular chondrocytes. It has been shown that for cells expanded in serum-containing medium, this process can be reversed by transferring cells to a suspension culture system in the presence of serum (Benya and Shaffer, Cell, 30:215-224, 1982). If cells expanded in DM in monolayer are intended for implantation for healing of cartilage defects (Brittberg *et al*, 1994), it is important to demonstrate they retain the potential to redifferentiate in suspension culture. A standard procedure for testing for redifferentiation potential is to suspend cells expanded in monolayer into agarose and test for deposition of sulfated glycosaminoglycans by staining with safranin-O.

A need exists to standardize and control the proliferation and differentiation of adult human articular chondrocytes (HAC) cultured for any medical application, especially for application in humans.

Summary of the Invention

One object of the present invention is based upon the development and use of a defined cell culture medium (serum-free) comprising a supplement mixture, a component mixture, a vitamin mixture, an inorganic salt mixture and amino acid mixture that avoids the problems inherent in the use of serum. In

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particular, the defined medium is useful in culturing fibroblasts, especially chondrocytes.

Another object of the present invention is to claim a method for enhancing the differentiation of chondrocytes and for enhancing the synthesis of a cartilage specific matrix using tumor growth factor beta (TGF- β).

Another object of the present invention is to claim a method for enhancing the differentiation of chondrocytes using a combination of TGF- β and IGF.

Detailed Description of the Invention

One aspect of the present invention is based upon the development of a defined cell culture medium and culture method to standardize and control the proliferation and differentiation of human articular chondrocytes (HAC) cultured for implantation into humans for repair of articular cartilage defects.

HAC were first cultured by plating the cells at 3000 cells per cm^2 and allowing them to attach for one day in Dulbeccos Modified Eagles Medium (DMEM) supplemented 10% serum, then removing the serum-containing medium and refeeding with DMEM basal medium in combination with a broad array of concentrations of the supplements described in Table 1. Every two to three days thereafter, cells were refed by completely replacing DM with fresh DM. Unable to induce cells to proliferate under these conditions, different basal media were tried and found that a 1:1:1 ratio of DMEM:RPMI 1640:Ham's F12 (DRF) when combined with the supplements was effective in promoting cell proliferation after plating as above. HAC cultured in DRF + supplements (complete DRF or cDRF) attained a terminal density equal or greater to the terminal density attained during culture in serum-supplemented medium. However, during the first several population doublings when the cell density was low, the proliferation rate was slow relative to the rate in 10% serum. We demonstrated cell proliferation without providing the cells with serum for one day prior to addition of cDRF. However, the initial growth of these cultures was slow and variable. Further experiments testing alternative supplements and different concentrations of the supplements failed to produce a cell culture system that consistently supported vigorous growth of HAC plated directly into DM without including serum at any step.

We had the idea that if we exchanged only half of the cDRF at refeeding, instead of completely exchanging the medium, factors secreted by the chondrocytes would boost their own proliferation. If the HAC secrete factor(s) required for growth in cDRF, when the cell density is sparse, the quantity of the secreted factor may be close to the threshold requirement. This would explain the slow growth in early cultures and the variable results observed in the above experiments. Using the cDRF medium combined with the approach of partial refeeding (described below), we obtained unexpectedly high yields of HAC in the complete absence of serum or any other undefined component after a brief time in culture. In the examples shown below, the volume of cDRF used was reduced relative to the volume of serum-rich medium because presumably this would increase the concentration of secreted factors in the media and further promote proliferation. Later experiments indicate that reducing the volume is not necessary and more optimal results may be achieved by using the larger volume while maintaining the practice of partial refeeding.

We have demonstrated that HAC cultured in suspension after expansion in cDRF generate colonies which stain positive with safranin-O. This indicates that, during expansion in DM, the cells have not lost their capacity to produce sulfated glycosaminoglycans, markers of chondrocyte differentiation. Because they retain their capacity to redifferentiate, chondrocytes expanded in cDRF may be suitable for autologous implantation for the purpose of healing cartilage defects.

Composition of cDRF:

The culture medium, named cDRF, is composed entirely of commercially available and chemically defined basal media and growth supplements. cDRF is a modification of the DM developed by Adolphe et al (1984). As supplements to the basal media, we have discovered that insulin transferin selenium (ITS) purchased from Collaborative Biomedical Products ((CBP) Bedford, Massachusetts), hydrocortisone purchased from Sigma (St. Louis, Missouri), basic fibroblast growth factor (FGF), fibronectin purchased from CBP and insulin growth factor (IGF), both available from Genzyme Corporation (Cambridge, Massachusetts), are particularly useful in achieving the objectives of the medium described in this disclosure.

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Table 1: Composition of cDRF

DMEM	33%
RPMI	33%
HAM'S F-12	33%
Supplements:	
ITS	1%
Penicillin &	100 U/ml
Streptomycin	100 ug/ml
Hydrocortisone	40 ng/ml
Basic FGF, human	10 ng/ml
IGF-I, human	1 ng/ml
Fibronectin, human	5 ug/ml

Method of preparation of cDRF:

All materials are reconstituted, diluted, and stored as recommended by supplier. The three basal media, DMEM purchased from Gibco BRL, Grand Island, New York, Cat# 11965-084 (Table 2), RPMI DMEM purchased from Gibco BRL, Cat# 11875-051 (Table 2) and Ham's F12 purchased from Gibco BRL, Cat#11765-021 (Table 2), are combined in a 1:1:1 ratio referred to hereinafter as DRF (Table 3). ITS, penicillin/streptomycin purchased from BioWhittaker, and hydrocortisone are diluted into DRF and this medium is stored up to 2 weeks at 2-8°C. Basic FGF, IGF, and Fibronectin are diluted into the complete DRF medium (cDRF) on the day of use for cell culture.

Table 2 Commercial Mediums

	DMEM	RPMI	HAM'S F-12
	1X Liquid mg/L	1X Liquid mg/L	1X Liquid mg/L
Inorganic Salts:			
CaCl ₂ (anhyd.)	200.00		33.22
Ca(NO ₃) ₂ •4H ₂ O		100.00	
CuSO ₄ •5H ₂ O			0.0024
Fe(NO ₃) ₃ •9H ₂ O	0.10		
FeSO ₄ •7H ₂ O			0.83
KCl	400.00	400.00	223.60
MgSO ₄ (anhyd.)	97.67	48.84	
MgCl ₂ (anhyd.)			57.22
NaCl	6400.00	6000.00	7599.00
NaHCO ₃	3700.00	2000.00	1176.00
NaH ₂ PO ₄ •H ₂ O*	125.00		
Na ₂ HPO ₄ (anhyd.)		800.00	142.00
ZnSO ₄ •7H ₂ O			0.86
Other Components			
D-Glucose	4500.00	2000.00	1802.00
Glutathione (reduced)		1.00	
Hypoxanthine•Na			4.77
Linoleic Acid			0.084
Lipoic Acid			0.21
Phenol Red	15.00	5.00	1.20
Putrescine 2HCl			0.161
Sodium Pyruvate			110.00
Thymidine			0.70

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Table 2 Commercial Mediums Continued

Amino Acids:			
L-Alanine			8.90
L-Arginine		200.00	
L-Arginine•HCl	84.00		211.00
L-Asparagine•H ₂ O			15.01
L-Asparagine (free base)		50.00	
L-Aspartic Acid		20.00	13.30
L-Cystine•2HCl	63.00	65.00	
L-CysteineHCl •H ₂ O			35.12
L-Glutamic Acid		20.00	14.70
L-Glutamine	584.00	300.00	146.00
Glycine	30.00	10.00	7.50
L-Histidine•HCl•H ₂ O	42.00		21.00
L-Histidine (free base)		15.00	
L-Hydroxyproline		20.00	
L-Isoleucine	105.00	50.00	4.00
L-Leucine	105.00	50.00	13.10
L-Lysine•HCl	146.00	40.00	36.50
L-Methionine	30.00	15.00	4.50
L-Phenylalanine	66.00	15.00	5.00
L-Proline		20.00	34.50
L-Serine	42.00	30.00	10.50
L-Threonine	95.00	20.00	11.90
L-Tryptophan	16.00	5.00	2.00
L-Tyrosine•2Na•2H ₂ O	104.00	29.00	7.81
L-Valine	94.00	20.00	11.70
Vitamins:			
Biotin		0.20	0.0073
D-Ca pantothenate	4.00	0.25	.50
Choline Chloride	4.00	3.00	14.00
Folic Acid	4.00	1.00	1.30
i-Inositol	7.20	35.00	18.00
Niacinamide	4.00	1.00	0.036
Para-aminobenzoic Acid		1.00	
Pyridoxine HCl		1.00	0.06
Pyridoxal HCl	4.00		
Riboflavin	0.40	0.20	0.037
Thiamine HCl	4.00	1.00	0.30
Vitamin B ₁₂		0.005	1.40

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Table 3 UKRr

Inorganic Salts:	
CaCl ₂ (anhyd.)	233.22
Ca(NO ₃) ₂ •4H ₂ O	100.00
CuSO ₄ •5H ₂ O	0.0024
Fe(NO ₃) ₃ •9H ₂ O	0.10
FeSO ₄ •7H ₂ O	0.83
KCl	1023.60
MgSO ₄ (anhyd.)	146.51
MgCl ₂ (anhyd.)	57.22
NaCl	19999.00
NaHCO ₃	6876.00
NaH ₂ PO ₄ •H ₂ O	125.00
Na ₂ HPO ₄ (anhyd.)	942.00
ZnSO ₄ •7H ₂ O	0.86
Other Components	
D-Glucose	8302.00
Glutathione (reduced)	1.00
Hypoxanthine•Na	4.77
Linoleic Acid	0.084
Lipoic Acid	0.21
Phenol Red	21.20
Putrescine 2HCl	0.161
Sodium Pyruvate	110.00
Thymidine	0.70
Amino Acids:	
L-Alanine	8.90
L-Arginine	200.00
L-Arginine•HCl	295.00
L-Asparagine•H ₂ O	15.01
L-Asparagine (free base)	50.00
L-Aspartic Acid	33.30
L-Cystine•2HCl	128.00
L-Cysteine HCl•H ₂ O	35.12
L-Glutamic Acid	34.70
L-Glutamine	1030.00
Glycine	47.50
L-Histidine•HCl•H ₂ O	63.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine	159.00

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Table 3 DRF Continued

L-Leucine	168.10
L-Lysine•HCl	222.50
L-Methionine	49.50
L-Phenylalanine	86.00
L-Proline	54.50
L-Serine	82.50
L-Threonine	126.90
L-Tryptophan	23.00
L-Tyrosine•2Na•2H ₂ O	140.81
L-Valine	125.70
Vitamins:	
Biotin	.2073
D-Ca pantothenate	4.75
Choline Chloride	21.00
Folic Acid	6.30
i-Inositol	60.20
Niacinamide	5.036
Para-aminobenzoic Acid	1.00
Pyridoxine HCL	1.06
Pyridoxal HCl	4.00
Riboflavin	0.637
Thiamine HCl	5.30
Vitamin B ₁₂	1.405

Articular cartilage was harvested from femoral condyles of recently deceased human donors (age range: 29 to 53) within 24 hours of death and stored in isotonic media for up to 4 days at 2-8°C. Chondrocytes (HAC) were released from the cartilage by overnight digestion in 0.1% collagenase/DMEM. Remaining cartilage was further digested for 4 hours in 0.1% collagenase/0.25% trypsin/DMEM. The released cells were expanded as primaries in DMEM supplemented with 10 % Fetal Bovine Serum, 100 U/ml Penicillin , and 100ug/ml Streptomycin (serum-rich medium). At near confluence, cells were frozen in 10%DMSO/40% serum/50%DMEM.

For experiments performed with 2nd passage cells, ampules of frozen primaries were thawed, rinsed in media indicated below for initial seeding. For experiments performed in 3rd passage, cells were expanded through 2nd passage

in serum-rich media, harvested by trypsinization, and washed in seeding media as indicated.

The following disclosure describes the use of collagen matrices and the cytokine TGF- β to enhance the redifferentiation and cartilage matrix formation process for dedifferentiated human articular chondrocytes. These findings are novel in that the application demonstrates how the cytokine augments the re-expression of the differentiated chondrocyte phenotype for passaged and dedifferentiated human cells in a matrix rather than simply supporting the differentiated phenotype for chondrocytes freshly released from cartilage tissue (primary cells) as others have shown (1,2). For those that have looked at the cytokine and its effect on re-expression, none have used it in a collagen matrix. The re-expression work centered on either rabbit cells in an agarose matrix (3,4) or else the factors effect on human chondrocyte proliferation (5) and not differentiation specifically. This disclosure is a first demonstration that the use of the cytokine can augment the redifferentiation of the cells and enhance the rate at which new cartilage specific matrix is synthesized in the collagen sponge environment. This should enhance the ability of this system to regenerate new tissue, support increased mechanical loads and reform the articular surface.

This disclosure describes a completely defined medium which will permit the re-expression of CII, a marker for chondrocyte differentiation, in a suspension of normal adult human articular chondrocytes that have de-differentiated as a consequence of expansion in monolayer *in vitro*. It has been discovered that TGF- β 1 or β 2 and IGF-I satisfy the growth factor requirement for this differentiation process. This combination of growth factors in defined medium is potentially applicable to improvements in the procedure of chondrocyte autologous transplantation (Bittberg et al, 1994). It may be used to prime chondrocytes for differentiation prior to implantation. Alternatively, it may be included as a supplement at the time of implantation of the chondrocytes. This growth factor combination may also be used as a differentiation-stimulating supplement to chondrocytes embedded in a matrix intended for implantation into cartilage defects.

Example 1:

To test the concept of partial refeeding with cDRF, we compared chondrocyte growth by this new method with growth in culture conditions which we were familiar with: culture in Fetal Bovine Serum (FBS) or culture in cDRF after one day in FBS with complete refeeding. Human chondrocytes from a 31 year old donor (HC31 cells) at 3rd passage were cultured under the conditions 1,2 & 3 described below.

Culture Condition 1 (FBS/complete refeeding):

Chondrocytes prepared as described above were seeded in triplicate into 10 cm² tissue culture wells at a density of 3,000 cells per cm² in 5 ml 10% FBS/DMEM and refed with 5 ml 10% FBS/DMEM one day after seeding and every 2-3 days thereafter. At each refeeding, all media was removed and replaced with 5 ml of fresh medium.

Culture Condition 2 (FBS-cDRF/complete refeeding):

Chondrocytes were cultured as for condition 1 except that after one day in 10% FBS/DMEM, all refedings were done with cDRF.

Culture Condition 3 (FBS-cDRF/partial refeeding):

Chondrocytes were cultured as for condition 1, except that after one day in 10% FBS/DMEM, all medium was removed and replaced with 3 ml cDRF. At each refeeding thereafter, partial refeeding was achieved by removing 1.5 ml of used cDRF and replacing with 1.5 ml of fresh cDRF.

Cells were harvested at 7 and 13 days after seeding and samples were counted on a hemacytometer. The results in Table 4 show a marked enhancement of cell yield at 7 days and at 13 days in conditions of partial refeeding with cDRF compared to that of complete refeeding.

Table 4: Example 1 results				
Exp 1 Culture conditions			Strain HC31, 3rd passage Cell density at harvest (1000's of cells/cm ² +/- sem)	
#	Day 0-1	Days 2-13	Day 7	Day 13
1	10% FBS	10% FBS Complete refeed	77 +/- 7	109 +/- 10
2	10% FBS	cDRF Complete refeed	29 +/- 1	72 +/- 13
3	10% FBS	cDRF Partial refeed	103 +/- 9	157 +/- 7

Example 2:

We repeated example 1 but added in one more condition to determine whether we could eliminate the use of serum during the first day after seeding:

Culture Condition 4 (cDRF/partial refeeding):

Chondrocytes were cultured as for condition 1, except that they were seeded in 3 ml cDRF instead of 5 ml serum-rich media. At each refeeding, 1.5 ml of used cDRF was replaced with 1.5 ml of fresh cDRF.

Cells were harvested at 7 days after seeding. The results in Table 5 are generally similar to the results of example 1 for the three culture conditions that were repeated. Interestingly, the additional culture condition, in which direct plating of cells into cDRF was combined with partial refeeding, yielded a yet higher quantity of cells.

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Table 5: Example 2 results			
Exp 2 Culture conditions			Strain HC31, 3rd passage Cell density at Day 7 (1000's of cells/cm ² +/- sem)
#	Day 0-1	Days 2-7	
1	10% FBS	10% FBS Comple te refeed	82 +/- 4
2	10% FBS	cDRF Comple te refeed	29 +/- 3
3	10% FBS	cDRF Partial refeed	73 +/- 2
4	cDRF	cDRF Partial refeed	126 +/- 2

Example 3:

We repeated example 2 with three additional human articular chondrocyte strains. The results in Table 6 show that partial refeeding consistently and substantially outperforms complete refeeding (condition 3 vs 2). Unexpectedly, plating directly into defined medium consistently outperforms attachment in serum, when partial refeeding is done in both cases (condition 4 vs 3). With the exception of one strain, partial refeeding with defined medium outperforms culture in 10% FBS with complete refeeding.

Table 6: Example 3 results						
Exp 3 Culture conditions			2nd passage, Cell density at Day 7 (1000's of cells/cm ² +/- sem)			
#	Day 0-1	Days 2-7	Strain HC31	Strain HC53	Strain HC29	Strain HC34
1	10% FBS	10% FBS Complete refeed	88 +/- 4	69 +/- 5	75 +/- 7	53 +/- 3
2	10% FBS	cDRF Complete refeed	24 +/- 3	40 +/- 1	9 +/- 3	6 +/- 0
3	10% FBS	cDRF Partial refeed	60 +/- 3	81 +/- 9	40 +/- 7	10 +/- 1
4	cDRF	cDRF Partial refeed	202 +/- 5	not done	88 +/- 4	27 +/- 4

Although there are only results for the 7 day timepoint in examples 2 & 3, the three examples combined are a strong indication that we can consistently attain cell densities of >100,000 cells/cm² within two weeks of culture by thawing frozen 1st passage cells, plating directly into defined medium without serum, and refeeding with half volumes.

Example 4:

We repeated example 2 again. In addition, we added one other condition to determine whether the partial refeeding method conferred an advantage to chondrocytes cultured in FBS:

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Culture Condition 5 (FBS/partial refeeding):

Chondrocytes were cultured as for condition 1 except that at each refeeding, half the media was removed and replaced with fresh media.

The results in Table 7 are again consistent with previous examples showing a clear advantage of partial refeeding over complete refeeding when cDRF medium is used. In contrast, when serum-rich media is used, the partial refeeding method does not increase and may decrease cell yields.

Table 7: Example 4 results			
Exp 4 Culture conditions			Strain HC31, 2nd passage Cell density at Day 7 (1000's of cells/cm ² +/- sem)
#	Day 0-1	Days 2-7	
1	10% FBS	10% FBS Complete refeed	77 +/- 2
2	10% FBS	cDRF Complete refeed	26 +/- 5
3	10% FBS	cDRF Partial refeed	67 +/- 11
4	cDRF	cDRF Partial refeed	103 +/- 3
5	10% FBS	10% FBS Partial refeed	49 +/- 2

Example 5

To assess the redifferentiation potential of chondrocytes after their expansion in monolayer culture in cDRF by the partial refeeding method, their capacity to form colonies in agarose which bind safranin-O (Saf-O positive colonies) was assessed. Strain HC31 chondrocytes prepared as described above were thawed and seeded at 2nd passage into 225 cm² tissue culture flasks (T225) at a density of 2,200 cells per cm² in 100 ml cDRF per T225. Cells in cDRF were refed by removing 50 ml (one-half the total volume) and replacing with 50 ml fresh cDRF. Refeeding was done one day after plating and every 2-3 days thereafter. As a positive control, parallel cultures were plated in 60 ml 10% FBS/DMEM per T225. Cells in 10% FBS/DMEM were refed by removing the full volume of used medium and replacing with 60 ml fresh 10% FBS/DMEM. Four T225s were plated for each condition.

Cells were harvested by trypsinization from two T225's per culture condition at 12 and 14 days after seeding. At harvest, the cells were suspended at 2.5×10^5 cells per ml in 10% FBS/DMEM and mixed 1:1 with 4% low-melt agarose. Four ml of the cell/agarose suspension were plated onto a layer of 2 ml solidified high-melt agarose in 60 mm tissue culture dishes (P60). Platings were done in duplicate or triplicate. After solidification, the cultures were overlaid with 5 ml 10% FBS/DMEM. The cultures were refed after 2-3 hours of equilibration and every 2-3 days thereafter until fixation.

After 3 weeks in agarose culture, the cells were fixed in 10% formalin and stained with safranin-O. Saf-O positive colonies of $\geq 2 \mu\text{m}$ diameter were counted using a microscope. For each corresponding monolayer condition, a total of 10 grids of 4 mm² each were counted randomly from 2 P60s.

The results in Table 8 show that the capacity of HAC expanded in cDRF to generate Safranin-O colonies after suspension in agarose is not statistically different than that of cells expanded in FBS. The similarity is clearer in the results from the 14 day monolayer cultures which have a smaller sampling error. The reduction in the number of colonies generated after 14 days in monolayer, either

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FBS or cDRF, may be the consequence of maintaining the cells in monolayer in a post-confluent state.

Table 8: Differentiation of HAC in agarose after expansion in cDRF or FBS		
Days in monolayer	Growth medium	# of safranin-O colonies ≥ 2 μm per 10 grids (40 mm²) 3 weeks after suspension in agarose (average of two cultures +/- s.e.m.)
12	10% FBS	436 +/- 31
	cDRF, partial refeed	327 +/- 96
14	10% FBS	203 +/- 38
	cDRF, partial refeed	249 +/- 20

Example 6

Primary chondrocytes were isolated from cartilage tissue from the femoral head of a 31 year old male. The cells were subcultured in monolayer. At third passage, the cells were seeded into a type-I collagen sponge matrix (Instat, Johnson&Johnson) at 10^7 cells/ml and cultured in DME media supplemented with either 10% fetal bovine serum (serum control), 1% ITS+ media supplement (serum free control) or ITS+ with TGF- β 1 at 1 or 5 ng/ml ("low dose" or "high dose", Collaborative Biomedical Products, Bedford, MA). The DME media is standardly available, and may preferably include high glucose without sodium pyruvate. Differentiation state of the cells was determined by gene expression analysis with RNase protection (Hybspeed RPA Kit, Austin TX.) using ³²P-labeled mRNA probes for type-I and type-II collagen and the cartilage specific proteoglycan Aggrecan.

Matrix deposition was studied by use safranin-O/fast green stain as well monoclonal antibody staining for collagen type-II and chondroitin sulfate.

mRNA analysis of monolayer cells demonstrates type-I collagen expression with trace type-II and Aggrecan expression for the chondrocytes at the time of seeding. Upregulation of type-II collagen with concurrent downregulation of type-I collagen expression was consistently observed for samples cultured in high dose TGF- β supplemented and serum control cultures. In serum free and low dose TGF- β conditions only modest type-I collagen downregulation is observed. This concurrent expression behavior for type-I and type-II collagens is consistent with re-expression of the differentiated chondrocyte phenotype. By 4-weeks, an enhanced level of re-differentiation as shown by RNase protection, was observed for samples cultured in high dose TGF- β culture over the other groups. 8-weeks there was extensive proteoglycan staining throughout the thickness of the matrix for samples cultured in high dose TGF- β conditions demonstrated by both immuno- and histologic staining. For low dose TGF- β and for serum and serum free controls, new matrix staining was relegated to the periphery or isolated pockets within the sponge matrix. This study demonstrates that passaged human chondrocytes can re-express their differentiated phenotype in the type-I collagen sponge environment. The level of re-differentiation was demonstrated both at the genetic expression and at the matrix deposition level. TGF- β modulated this process by enhancing the rate of redifferentiation and the amount of new matrix deposition.

Example 7

Confluent or near-confluent third passage adult human femoral condyle chondrocytes were harvested by trypsinization and suspended in alginate beads at a density of 10^6 cells/ml. For each example described below, cells in alginate were cultured at 37°C, 9% CO₂, in 25 mM HEPES buffered DMEM supplemented with 100U/ml penicillin, 100 μ g/ml streptomycin (basal medium), and additional supplements as indicated. Storage and dilution of supplements were performed as recommended by suppliers. For each culture, 8 ml of alginate beads (8 million cells) were incubated in a 150 cm² flask in 40 ml of the indicated media. Cultures were re-fed every 2-3 days. At timepoints indicated, cells were released from alginate,

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pelleted, frozen and stored. RNA was isolated from the cell pellets and quantitated. The effect of the different culture conditions on the abundance of for collagen type I (CI), collagen type II (CII), aggrecan (Agg) mRNAs was determined by the Rnase protection assay, using 18S rRNA (18S) detection as an internal standard. The RNA probes used in the Rnase protection assay were transcribed from templates containing cDNA segments of the human genes for CI, CII, Agg, and 18S. The culturing of cells in alginate was done according to Guo, et al., Connective Tissue Research, 19: 277-297, 1989. The isolation of RNA was done according to manufacturer's instructions using the Qiasredder™ and RNeasy™ kits purchased from Qiagen (Chatworth, California). Rnase Protection assays were performed according to manufacturers instructions using Hybspeed™ RPA kit purchased from Ambion (Austin, Texas).

Alginate culture and RNase protection assay protocols

OUTLINE

- I. Expansion of chondrocytes in monolayer (1-2 weeks)
- II. Culture of chondrocytes in alginate (1 week-6months)
 - A. Inoculation of cells into alginate beads
 - B. Refeeding
 - C. Harvesting cells from alginate beads

STOP POINT

- III. Isolation, quantitation and aliquoting of cellular RNA (1-2 days; 1 day per set of RNA preps)
 - A. Isolation
 - B. Quantitation
 - C. Aliquoting

STOP POINT

- D. Visualization of RNA samples on agarose gels (evaluation of RNA degradation)

STOP POINT

IV. RNase protection assays (1-5 days)

- A. Preparation of specific cDNA templates with T7 promoter in antisense orientation (performed by FB)

Day 1:

- B. In vitro transcription from cDNA to prepare antisense radioactive RNA probes from cDNAs, followed by Dnase treatment to remove cDNA template (should be done within 3 days of Hybridization step, preferably the day before)
- C. Gel purification of radioactive probes
- D. Co-precipitation of cellular RNA with antisense probes (should be done the day before Hybridization step)

Day 2:

- E. Hybridization of cellular RNA with antisense probes and RNase treatment to remove ssRNA
- F. Electrophoresis of protected RNA
- G. Quantitation on phosphorimager

For R& D studies of chondrocyte differentiation, we cultured chondrocytes in alginate beads followed by detection of chondrospecific gene expression using RNase protection assays. The procedures, written in detail below, are derived from the following sources;

Alginate culture: Guo et al (1989) Connective Tissue Research 19:277-297

RNA isolation: Handbook from RNeasy Total RNA Kit (Qiagen, Cat # 74104)

In Vitro Transcription: Instruction Manual from MAXIscript T7 In Vitro Transcription Kit (Ambion, Cat # 1314)

RNase Protection Assay: Instruction Manual from HybSpeed RPA kit (Ambion, Cat # 1412)

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DETAILED PROTOCOLS**I. Culture of chondrocytes in alginate (1 week-6 months)****A. Inoculation of cells into alginate beads****Materials**

Monolayer cultures of chondrocytes, >8 million cells per 8 ml alginate culture to be inoculated PBS, 20ml/T150

Trypsin/EDTA (T/E), 20 ml per T150

DMEM 10% FBS for washing trypsinized cells, 30 ml per T150

0.15 M NaCl/25 mM HEPES, pH 7.4 (Isotonic Salt Solution),

~ 30 ml per T150 flask of monolayer cells+ ~ 200 ml per 8 ml alginate culture

1.2% alginate/0.15% NaCl/25 mM HEPES, pH 7.4, warmed to RT, 8 ml per alginate culture

Recipe (per 100 ml):

Position a glass beaker containing 100 ml Isotonic Salt Solution under a Polytron mixer. Insert the end of the Polytron in the solution and run at high speed while very slowly adding 1.2 grams of alginate (Improved Kelmar, from Kelco) and moving the beaker. After the alginate appears to be complete dissolved (~10-15 minutes), add magnetic stirbar and stir for ~30 minutes. Autoclave 30 minutes, then run through 0.45 micron filter then 0.22 micron filter and store for up to six months in the refrigerator.

0.1 M CaCl₂/25 mM HEPES, pH 7.4, 80 ml per 8 ml alginate culture

Medium for culture of chondrocytes in alginate, 40 ml per 8 ml alginate culture

Note: the basal medium affects the stability of the alginate beads; if using something other than DME as basal media, preliminary tests need to be done to test bead stability. See Guo et al (1989) Connective Tissue Res. 9:277

22 gauge needles and 10 ml syringes, one per alginate culture

25mM HEPES -buffered DMEM, 120 ml per 8 ml alginate culture

T75 tissue culture flasks, one per alginate culture

Plastic bottle for suspension of cells in NaCl, one for each set of alginate cultures, maximum of 6 alginate cultures per set (capacity of ~50-60 ml per monolayer T150 culture).

125 ml bottles, 1 per alginate culture

70 micron filters, 1 per alginate culture

Procedures

To avoid prolonged exposure of cells to 0.1 M CaCl_2 , prepare only ~6 alginate cultures (~48 million cells) at one time from monolayer culture

1) Prepare all materials listed above and prewarm EXXXX and growth media

2) warm T/E, 20 ml per T150 to be harvested

3) Aspirate media from each T150 monolayer flask, add 20 ml PBS to each, and aspirate

4) Add 20 ml T/E per T150, incubate ~2 minutes, suspend cells, rinse bottom of flask with 30 ml DMEM 10%FBS

5) Transfer to conical tube, pellet cells, resuspend each in 25 ml Isotonic Salt Solution, and combine into one plastic bottle

6) Rise the empty tubes in series with 30 ml Isotonic Salt Solution and add to cell suspension

Note total volume of cell suspension

7) Take ~1ml sample of cells to count on hemacytometer and fill four hemacytometer wells

8) RECORD cell yield and density of monolayer cultures at time of harvest

9) distribute 8 million cells into each 50 ml conical tubes (one per alginate culture or timepoint) and pellet 8 minutes at 1000 rpm

10) From one tube, aspirate sup, label T=0, immerse in liquid nitrogen, and freeze cell pellet immediately at -80°C (for RNA sample of monolayer culture)

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11) add 8 ml 1.2% alginate solution to each remaining cell pellet and resuspend by pipetting up and down about 30 times (do not introduce air bubbles.)

12) For each cell/alginate suspensions:

(i) mix again by pipetting up and down five times and transfer the suspension into a 10 ml syringe fitted with a 22 gauge needle, using a 10 ml pipette.

(ii) cover with plunger, invert, and remove air by depressing plunger and tapping to remove bubbles

(iii) cover with plunger, invert, and remove air by depressing plunger and tapping to remove bubbles

(iv) allow alginate beads to cure for 5-15 minutes at RT, no longer than this.

(v) pour CaCl_2 through 70 μm filter into waste bucket, and wash 2X with 100 ml Isotonic Salt Solution, and 2X with 60 ml DMEM using 70 μm filter and waste bucket

13) Resuspend each tube of beads into 20 ml of respective media and transfer to labelled T162, then rinse remaining beads into flask with another 20 ml. (~8 million cells per T162, or somewhat less as ~1 ml is lost during transfer into syringe)

14) place loosely capped flasks in incubator.

B. Refeeding, every 2-3 days

For each alginate culture:

1) Stand flask on end, and tilt to allow beads to settle in one corner of flask.

2) With 50 ml pipet, remove media from above the beads (~25-30 ml)

3) Place 50 ml pipette tightly against bottom end of the flask, and slowly lay flask flat on its end.

4) Withdraw all media from flask into pipet, maintaining contact between pipet tip and bottom of flask to avoid drawing beads into pipet.

- 5) Lift pipet above surface of media and examine for beads settling to tip of pipet. Dispel any beads that may be settled in pipet tip.
- 6) Discard media into bucket
- 7) Add 40 ml fresh prewarmed media to beads
- 8) Repeat for next culture. USE SEPARATE PIPET FOR EACH CULTURE

C. Harvesting cells from alginate beads and:

- i) determine cell yields
- ii) snap freeze cell pellets for RNase protection assay
- iii) prepare cytopsin slides for antibody staining

Materials, quantity per alginate culture

labelled 50 ml conical tubes, 1

50 ml pipettes, 1

70 micron-pore filters, 1

0.15 M Na Cl/25 mM HEPES, p H 7.4-175 ml

55 mM Na Citrate/100mM Na Cl/25 mM HEPES< p H 7.4~45 ml

microfuge tubes for samples for counting (labelled for each culture), 1

Trypsin/EDTA std working solution, 100µl

hemacytometers, 1

labelled 15 ml tubes for cytopsin aliquots, 1

cytopsin loading and slide assembly, labelled ≥6

4% paraformaldehyde, 200 ml (use within one week, store tightly capped at 4°C)

Note: paraformaldehyde is toxic

To prepare 200 ml:

i) in fume hood, warm 200 ml PBS+200 µl 2N Na OH to 70-75°C on hot stir plate; do not heat above 80°C.

ii) Turn off heat, add 8 g paraformaldehyde and stir with magnetic stir bar until solution becomes clear (less than one hour)

Be sure to clean up any paraformaldehyde dust.

iii) test pH; pH should be between 7 & 7.5

iv) filter through Whatman paper #1 in fume hood

70% EtOH, 200 ml

PBS, 200 ml

Holders for submerging cytopsin slides

Holders for storing cytopsin slides

Methods

1) photograph beads under microscope

2) transfer cells of each flask to separate 50 ml conical tube, using 30 ml pipets

3) wash 3X WITH 0.15M NaCl/25 mM HEPES

i) Drain liquid into waste bucket using 70 micron filter

ii) Add 0.15M NaCl/25 mM HEPES to 45 ml mark on conical tube by pouring

iii) Repeat draining and pouring 2 more times, and drain once more.

4) add 55 mM NaCitrate/100 mM NaCl/25 mM HEPES to 50 ml mark and repeatedly invert gently for 5-8 minutes (for ~one minute after beads become undetectable by eye).

5) centrifuge 8 min at 1000 rpm in tabletop IEC (or Mistral) centrifuge

6) aspirate sup and resuspend in 20 ml 0.15 M NaCl/25 mM HEPES.

7) mix well, transfer 100 μ l into microfuge tube containing 100 μ l T/E for counting (step 11) and place tubes in 37°C water bath

8) count cells (from step 7, two hemacytometer wells/sample)

—cell samples from cultures not to be put on cytopsin can be counted after last step

RECORD CELL COUNTS

cell yield per culture+(counts/5 fields)

$(20)(2)(10^4)/5 = (\text{counts}/5\text{fields})(80,000)$

9) aliquot cells from each culture into 15 ml tubes for cytopsin, enough for 50,000 cells per slide, at least six slides per culture

- 10) add 30 ml 0.15 M Na Cl/25 mM HEPES to step 6 suspension
- 11) centrifugation at 1000 rpm for 8 minutes
- 12) aspirate sup and IMMEDIATELY freeze cell pellet at -80°C

Ideally, it is better to snap-freeze cells in liquid nitrogen or dry ice/ethanol bath, and then move to -80°C for storage. Slow freezing may lead to degradation of cellular RNA

- 13) dilute aliquots of cells for cyospin to 50,000 cells/500 µl
- 14) load 500 µl sample (50,000 cells) into each cyospin loading device (at least six samples per cell culture)
- 15) Spin cyospin devices at 800 rpm for 5 minutes (Program #1)
- 16) Air dry for 1-2 minutes in tissue culture hood
- 17) Fix in 4% paraformaldehyde for 5 minutes
- 18) Drain slides and transfer to PBS for 2 minutes
- 19) Drain slides and transfer to 70% EtOH for 3 minutes
- 20) Air dry in hood for 15 minutes or until completely dry.
- 21) Store at -80°C. Upon thawing, fix in 4% formaldehyde for 2 min and rinse with PBS.

STOP POINT: RNA within cell pellet stored at -80°C should be stable "indefinitely"

III. Isolation , quantitation, aliquoting, and get electrophoresis of cellular RNA (1-2 days; 1 day per set of RNA preps)

Materials for isolation, quantitating, and aliquoting, quantity per RNA prep

Gloves

Autoclaved, labelled microfuge tubes, 1

RNeasy Total RNA Kit, Qiagen

Lysis buffer RLT: 600ul

2 ml collection tubes, labelled:1

spin columns in 2 ml collection tubes, 1

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Wash buffer RW1: 700 μ l

Wash buffer RPE concentrate: 200 μ l (or 1 ml if already diluted with ethanol)

1.5 ml collection tubes, labelled: 1

Beta mercaptoethanol (BME), 6 μ l

QIA shredder in 2 ml collection tube (Qiagen), 1 units

70% ethanol/30% DEPC-treated H_2O , 600 μ l

100% Ethanol, 800 μ l (for diluting RPE concentrate, unless RPE is already diluted)

DEPC -treated dH_2O /0.1mM EDTA, 50-200 μ l (depending on yield of RNA)

Rnase-free pipet tips with aerosol barrier for P2, P20, P200 and P1000

650 μ l presiliconized, Rnase-free microfuge tubes (Sorenson), 5

Screw-cap microfuge tubes for storage: 1

Spectrophotometer and UV Silica Ultra Microcell (Beckman Cat# 514261)

Capillary UV "cuvettes", 0.5 mm pathlength (Beckman Cat# 514262)

Procedures

A. Isolation

1) Review notes p7 of RNeasy handbook (attached); This includes important notes regarding prevention of RNA degradation, handling of kit components, and limitations of the RNeasy kit

2) Label tubes/columns for tracking of RNA samples throughout procedure; 1.5 ml autoclaved microfuge tubes for initial transfer of cells/lysis buffer suspension, 1 per RNA sample

QIA shredder columns in 2 ml collection tubes, 1 per RNA sample

RNeasy spin columns in 2 ml collection tubes, 1 per RNA sample

2ml collection tubes from RNeasy kit, 1 per RNA sample

1.5 ml collection tubes from RNeasy kit, 1 per RNA sample

650 μ l Rnase free presiliconized tubes for storage of RNA aliquots in dH_2O /EDTA, 5 per RNA sample

screw-cap microfuge tubes for storage of RNA in Et-OH, 1 per RNA sample

Label container for storage of samples at -80°C

3) Add 10 µl BME per ml Lysis Buffer RLT from RNeasy kit
Lysis Buffer RLT may form precipitate upon storage. Warm to redissolve.

4) **Lysis and homogenization of cells:** (RNeasy step 1b and 2)
Remove cell pellets from -80°C (See Harvesting cells from alginate beads)

Immediately add 600 µl RLT buffer + BME (step 4) directly to each cell pellet; allowing cells to thaw without first adding lysis buffer can lead to RNA degradation.

Mix by pipetting and transfer to labelled 1.5 ml microfuge tubes.

Vortex each tube 30 seconds at high speed

Spin in microfuge momentarily

Mix briefly with pipet and transfer to QIA shredder

Spin at full speed in microfuge for 1 minute. If insoluble materials is visible in lysate (flowthru), microfuge lysate for 3 minutes at full speed and use only the supernatant for remaining steps. This second microfugation has not been necessary to date.

5) **RNeasy step 3:**

Add 1 volume (600 µl) 70% ethanol/DEPC-dH₂O per sample and mix by pipetting. This lysate must not be centrifuged.

6) **Application of cell lysate to spin column:** (RNeasy step 4)

Transfer 600 µl of lysate to RNeasy spin column. Microfuge 15 seconds at 10,000 rpm (~8,000 X g). Discard flow-thru. Transfer remaining lysate into the same column and microfuge as above.

7) **Purification of RNA in Spin Column:** (RNeasy step 5)

Add 700 µl Wash Buffer RW1 into spin column, centrifuge as above and discard flow-thru. According to the trouble-shooting guide (p 23 of Rneasy manual), allowing the column to sit for 5 minutes after

addition of RW1 and before centrifuging may reduce DNA contamination

8) Further purification of RNA in Spin Column: (RNeasy step 6 & 7):

Combine 1 volume of Wash Buffer RPE concentrate (RNeasy kit) with 4 volumes of 100% ethanol (RT), unless RPE buffer has already been diluted with ethanol

Transfer the spin column used above into a new 2 ml collection tube; RNA is still in column. Add 500 µl of Wash buffer RPE/Ethanol (1:4) to spin column.

Microfuge as above

Discard flow through

Add 500 µl Wash buffer RPE/Ethanol (1:4) again to same spin column

Microfuge 2 minutes at full speed

Discard flow thru and collect on tube. Inspect for ethanol on outside of spin column and remove with kimwipe if necessary. Residual ethanol may interfere with subsequent steps. Transfer the spin column used above to a 1.5 ml collection tube supplied with kit.

9) Elution of RNA from spin column: (RNeasy step 8)

Carefully add 30 µl DEPC-treated water/0.1 mM EDTA per sample directly to membrane of the spin column, without touching the membrane with the pipet tip but making sure that the entire membrane is wetted

Microfuge for 60 seconds at 8.000 X g

Repeat above elution, using 20 µl DEPC-treated water/0.1mM EDTA and using the same spin column and collection tube, to yield an RNA eluate totaling 50 µl

Place all samples on ice during Quantitation and aliquoting.

B. Quantitation of RNA by UV absorbance at 260

- 1) Transfer 3 µl each RNA sample 57 µl DEPC-treated H₂O (20-fold dilution)

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- (2) Read samples against DEPC-treated H₂O blank in short (60 μ l volume) 1 cm pathlength quartz cuvette (available on 4th flr. 1 MTN RD) at 260 and 280 nm.

RECORD readings

1 O.D unit @ 260 nm, 1 cm pathlength + 40 μ g/ml RNA, and translates to 800 μ g/ml after accounting for the 20-fold dilution. This reading would correspond to a total yield of 40 μ g for a 50 μ l sample.

My yields have typically ranged from ~8-80 μ g from 8 ml alginate cultures, depending on the conditions of culture. However, since I have done these preps, the methods for harvesting cells from alginate have been modified with the intention of improving cell yield (specifically, the g-force during centrifugation of cells after dissolving alginate beads has been increased ~5-fold).

A_{260}/A_{280} + 1.7 to 2.0 indicates "highly pure" RNA, 2.0 is ideal. Lower ratios indicate the presence of protein contamination. Typically, the ratio is near 2.0. Readings at 320 nm indicate presence of carbohydrate.

C. Aliquoting

- 1) For RNA concentrations above 500 μ g/ml, dilute to 500 μ g/ml by adding DEPC-dH₂O/0.1mM EDTA. Record final concentration.
- 2) For each RNA sample, calculate the volume that is equivalent to 1.0 μ g.
- 3) Make 5 aliquots of 1.0 μ g each into 650 μ l presiliconized, Rnase-free microfuge tubes.
- 4) Transfer remaining RNA, if any, to screwtop Rnase-free 1.5 ml microfuge tubes, add 1/10th volume 1% SDS and \geq 2.5 volumes 100% Et-OH, vortex, and store at -80°C.
- 5) Record all aliquot volumes and concentrations
- 6) Store all aliquots at -80°C

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Notes:

i) 1.0 μ g RNA aliquots in DEPC-dH₂O/0.1mM EDTA: For visualization of RNA on agarose gels or for RNase Protection Assays, 1.0 μ g or less RNA is adequate. Because of the volume of the aliquots may be small (as little as 2 μ l) and because of possible dessication of samples in freezer, do not subaliquot the 1.5 μ g aliquots stored in aqueous solution until they have been diluted into larger volumes after thawing. Use the 1.0 μ g aliquots once and discard any unused RNA after thawing. According to the RNeasy Handbook, RNA stored in water at -80°C, or even -20°C, should be stable for at least a year.

ii) Remaining RNA stored in Ethanol: The samples stored in Ethanol should allow longer-term stability. This RNA must be treated as a suspension, not a solution. Before taking any sub-aliquots from these suspensions, they must be thoroughly vortexed.

Ideally, it is better to precipitate the RNA, remove the ethanol, redissolve in aqueous solution, and requantitate before using.

STOP POINT

D. Visualization of RNA samples on agarose gels (evaluation of RNA degradation)Materials

Agarose, Molecular Biology Grade (SeaKem GTG), 0.4 g per gel, 14 samples per gel

37% formalin,

Autoclaved dH₂O, ~250 ml per gel

10X MOPS,

Components:

0.2M MOPS

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50mM sodiumacetate

10mM EDTA

pH should be 7

autoclaved

Electrophoresis apparatus and tray (~30 ml gel capacity), combs (14 teeth, thick), power supply

RNA samples, one 1.0 μ l aliquot each stored at -80°C, up to 13 samples per gel

RNA ladder, 0.24-9.5Kb @ 1 μ g/ μ l, 2 μ l per gel (~0.33 μ g of each band)

If precipitation is necessary:

DEPC-H₂O/0.1 mM EDTA

DEPC-H₂O/0.1mM EDTA, 3M NaAcetate, pH 5.2 ~50 μ l

100% Et-IH, RT

Vacuum dessicator

Heating block for 650 μ l tubes, 65°C

long-tipped pipetman tips and pasteur pipet (for drying RNA pellets)

1X RNA loading buffer, up to 12 μ l per sample

Recipe

0.75 ml formamide (stored at -200°C)

0.15 ml 10X MOPS

0.24 ml 37% formaldehyde

0.1 ml dH₂O, Rnase free

0.1 ml glycerol

80 μ l 10% (w/v) bromophenol blue

1 mg/ml EtBr,

camera/polaroid film/UV box

Procedures

Agarose gel preparation

1) Prepare 40 ml 1% agarose/1.9% formaldehyde/1X MOPS gel:

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Mix 34 ml autoclaved dH₂O + 4 ml 10X MOPS + 0.4 g of agarose

Microwave to dissolve agarose and cool to 50°C

In fume hood, add 2 ml 37% formaldehyde, gently mix, and pour into clean tray with comb

Allow gel to set for ~30 minutes, adding running buffer as soon as gelling occurs

2) Prepare 250 ml Running buffer per gel: 1X MOPS/1.9%

formaldehyde

212.5 ml dH₂O

25 ml 10MOPS

12.5 ml 37% formaldehyde

Preparation of RNA samples for electrophoresis

3) For 1 µg samples which are 8 µl or less (> 125 µg/ml),

Combine 1 µl Ethidium bromide per 18 µl 1X RNA Loading Buffer, and add 1X RNA Loading Buffer/EtBr mixture to a final volume of 24 µl.

For all other samples, precipitate (see next step).

4) For 1 µg samples that are more than 4 µl, it is necessary to concentrate samples by ethanol precipitation:

i) To each RNA sample, dilute to final volume of 18 µl with DEPC-H₂O/0.1 mM EDTA

ii) Add 2 µl 3 M Na Acetate, vortex, and add 50 µl 100% Et-OH to each sample.

iii) Vortex again and microfuge at full speed, RT, for 15 minutes. Be sure to orient tube such that you know where the RNA pellet will be.

iv) Aspirate Sup with long pipet tip, avoiding pellet. Microfuge 30 seconds full speed to collect residual ethanol, aspirate sup, and dry for 4' in vacuum dessicator.

v) Combine 1 µl Ethidium bromide per 23 µl 1X RNA Loading Buffer, and add 24 µl to each pellet

5) RNA Ladder: Make one 2 µl aliquot of RNA Ladder for each gel to be run and add 10 µl 1X RNA Loading Buffer

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6) Heat all of the RNA samples, including RNA Ladder at 65°C for 1 minute in heating block, vortex at setting 4-5 for 20 seconds, microfuge momentarily if sample splashes onto side of tube, heat at 65°C for 15 minutes, and snap cool on ice.

Gel Electrophoresis

8) microfuge momentarily, and load 12 µl of each sample per well, loading RNA Ladder into one well per gel. The quantity loaded will be 0.5 µg per well except the RNA ladder which will be 2 µg per well (~0.33 µg/band). Freeze remainder of samples in case samples need to be rerun.

9) Run gel:

100 Volts, -85 mA

Run ~ 1hr (run until bromophenol blue runs ~2/3 of gel distance)

10) Photo gel on UV box using photobox apparatus with shield (1/8 second, f-stop=4.5)

Save photo for notebook. The 18S and 28S rRNAs should be clearly visible. Obvious downward smearing of the rRNA bands is indicative of RNA degradation. The intensity of the bands should be comparable among samples, if quantitation and aliquoting were done properly.

STOP POINT

IV RNase protection assays (1-5 days)

A. Preparation of specific cDNAs templates with T7 promoter in antisense orientation:

Francois Binette, using PCR technology, has synthesized all the human cDNA template used in these studies, with the exception of the 18S rRNA gene which is supplied by Ambion. These cDNAs are linked to ~20 bp of the T7 phage promoter oriented to promote synthesis of radioactive RNA from

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the human genes in the antisense orientation upon addition of T7 RNA polymerase and radioactive nucleoside triphosphates. Francois' maps of the human genes showing the probe positions and sizes are attached.

The human cDNA templates currently available for these experiments include portions of the genes for :

Collagen Type I: chondrocyte dedifferentiation marker

Aggrecan, Collagen Types II & IX: chondrocyte differentiation markers

Collage Type X: chondrocyte hypertrophy marker

Day 1:

B. In vitro transcription from cDNA to prepare antisense radioactive RNA probes from cDNAs, followed by Dnase treatment to remove cDNA template.

--should be done within 3 days of Hybridization step, preferably the day before

See MAXIscript™ (Ambion) Instruction Manual for additional information on background, kit components, additional procedures, and troubleshooting.

The Rnase Protection Assay can be done using several probes combined, as long as the probes are different enough in size to allow separation during electrophoresis. However, each probe must be transcribed in separate tubes and gel purified from different lanes of the gel before combining for the Rnase protection assay. In addition to preparing probes from the templates listed above, the 18S rRNA template should also be transcribed for every Rnase protection assay. Presumably, the quantity of 18S rRNA is equivalent among cells, independent of growth conditions, and is therefore a standard for comparing the amount of total cellular RNA used in the Rnase Protection Assays.

Size marker RNA should also be used in each RNase Protection Assay. Unlike the other probes, these can be transcribed as much as 2 months in advance of use and do not need to be gel-purified.

Materials, quantity per probe

From MAXIscript™ T7 (Ambion, Cat #1314) in vitro transcription kit (FOR KITS

RECEIVED AFTER 11/1/95):

10X transcription buffer, 2 μ l

ATP solution, 10 mM, 1 μ l

GTP solution, 10mM, 1 μ l

UTP solution, 10mM, 1 μ l

T7 RNA polymerase (5 U/ μ l) + RNase Inhibitor (5 U/ μ l), 2 μ l

DNase I (RNase free), 2 U/ μ l, 1 μ l

2X Gel Loading buffer, 22 μ l:

CTP solution from Ambion MAXIscript kit diluted to 0.05 mM, 3 μ l

[alpha-32P]-CTP, 3000 Ci/mmole, 10 mCi/ml, 5 μ l (only 1 μ l for transcription of size markers) This should be ordered for delivery within 3 days of use (Delivery dates are Monday and Friday).

pT-7-Human cDNA templates (from 100 μ l PCR rxn performed by FB), 5 μ l (~0.5-1.0 μ g) each pT7 18S rRNA antisense control template, 0.5 μ g/ μ l (Ambion Cat # 7338) ("R"), 2 μ l total pT7 RNA size marker template, 1 μ l (unless transcribed within the last 2 months) DEPC-treated H₂O, up to 13 μ l RNase-free, screw-top 1.5 ml tubes, labeled for each probe, 1 Heating blocks for 1.5 ml tubes, one at 37°C and one at 95°C
Plexiglass radiation shield

Procedures

- 1) Set one heating block to 37°C and the other to 95°C
- 2) Move [alpha-32P]-CTP from freezer to RT
- 3) Thaw all reagents in table below to RT, except Polymerase and DNase I
- 4) Briefly vortex and microfuge xcription buffer and XTP solutions
- 5) To RNase-free 1.5ml tubes, add components in order shown below (#'s represent ul)

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All components, except the templates and polymerase can be combined as one batch, then distributed as 13 μ l aliquots, one to each tube to receive template. Then add one template per tube, then polymerase

--All procedures beginning with the addition of 32 P-CTP must be done using a plexiglass radiation shield

--Combine components at RT, NOT on ice

<u>Component(see above)</u>	<u>μl/probe</u>
10X xscription buffer (kit)	2
ATP (kit)	1
GTP (kit)	1
UTP (kit)	1
CTP (diluted from kit, FB)	3(for transcription of size marker, use 1 μ l of undiluted CTP)
32 P-CTP	5(for transcription of size marker, use 1 μ l)
Human cDNA templates	5 each
18S rRNA gene template	2 μ l template + 3 μ l DEPC-H ₂ O
*Size marker template	1 μ l template + 10 μ l DEPC-H ₂ O
T7 RNA Polymerase (kit)	2

After adding polymerase, mix components by pipetting

*Size marker does not need to be transcribed if some has already been prepared in

the last 2 months and is still available. There is no need to gel purify size

markers after the in vitro transcription reaction.

6) Incubate in 37°C heating block for 30-60 minutes

--During this period, prepare 4% acrylamide gel (See section C below)

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- 7) Add 1 μ l of DNase (kit) to each reaction, mix by pipetting, and incubate 15 minutes at 37°C
- 8) Add 21 μ l 2X Gel loading buffer (kit) and heat to 95°C for 2-3 minutes
 - prior to this step, gel should be ready; prolonged heating may lead to formation of aggregates in SDS that are not able to enter the gel during electrophoresis.
 - leave tubes in heating block for loading gel directly from block.
 - If size markers were transcribed, they can be frozen at this time; no need for gel purification.

C. Gel purification of radioactive probes

Materials

Marathon Gel Mix 4(premix for 4% acrylamide, 8.3M Urea gel), 15ml per two gels

Ammonium Persulfate (APS), 10% (prepared within one day), 90 μ l

Running buffer; 1X TBE, ~300 ml

Gel pouring apparatus

Comb with 40 μ l/well capacity (10 teeth, 0.75 mm thick)

Gel electrophoresis apparatus in radiation room

Fine tip pipetman tips

Electrophoresis power supply in radiation room

Plastic wrap

BIOMAX MR film (Kodak, Cat #895 2855)

RNase-free 1.5 ml tubes, 1 per probe + 1 for combined probes

Elution buffer (0.5M Ammonium Acetate, 0.2% SDS, 1mM EDTA), 300 μ l per probe

Clean single-edge razor blade

Scintillation fluid (Optiphase "HiSafe"), 5 ml per probe

Scintillation vials, 1 per probe

Scintillation counter, programmed for counting P32

Procedures

- 1) Clean and assemble gel pouring apparatus
- 2) Warm Marathon Gel Mix 4 to RT, add APS, swirl the solution and pour gel using 10 ml pipet
- 3) Insert comb, clamp, and add more gel solution to be sure gel reaches top of teeth on comb. Gel can sit for hours in gel pouring apparatus before proceeding
- 4) After gel sets (within 30 minutes), remove plate-gel-backing unit from apparatus and rinse plates with tap distilled water. Remove comb and rinse wells 2-3 times, shaking out water between rinses
- 5) Install plate-gel-backing in electrophoresis apparatus
- 6) Add 1X TBE to top reservoir, checking for leaking, and then to bottom reservoir
- 7) Just before adding samples from in vitro transcription (Section I), rinse wells with buffer in top reservoir, using P1000
- 8) With samples in 95°C heating block, load 30 µl of each sample into each well, spacing samples with empty wells
- 9) Hook up power supply and run gel at 125 V for ~ 1 hr or until fast dye is approx 2/3 down gel. Power may be raised to 200 V to speed electrophoresis
- 10) Remove plate/gel/backing from electrophoresis apparatus and rinse all components thoroughly with tap water.
- 11) Remove glass plate, leaving gel attached to backing
- 12) Wrap gel/backing with plasticwrap and expose x-ray film for 60 seconds, noticing orientation of gel and film (gel precisely in upperleft corner of film; dull side of film should be separated from gel by a single layer of plasticwrap)
- 13) Develop film, and using film as a guide, mark the location of the four bands containing the respective probes on the gel by adding mark directly to plasticwrap over gel with marking pen. Save film for X-ray film binder
- 14) With razor, cut out each band of the gel containing probe of interest, peel away plasticwrap, and drop each band into a separate 1.5 ml RNase-free tube of 300 µl Elution buffer
- 15) Incubate gel in elution buffer for 2.5-3 hrs at 37°C, then microfuge 1 minute at full speed
- 16) Add 10 ml scintillation fluid to each of four labeled scintillation vials

- 17) Add 3 μ l (1% of total volume) from each elution to separate scintillation vials
- 18) Count radioactivity in scintillation counter on ^{32}P program; Record CPM
- 19) Calculate the volume in μ l, for each probe, equivalent to 25,000 counts
- 20) Combine probes into one 1.5 ml RNase-free tube, using above calculations to give total of 25,000n CPM of each probe, where n equals the number of RNase protection assays (including controls) to be done with the probe mixture.
Because the energy from radiation can cause chemical breakdown of the RNA, the probes should be used within the next three days, the sooner the better
- 21) Return unused individual probes to freezer designated for radioactive materials.
Use shielded container.

D. Co-precipitation of cellular RNA with antisense probes

--should be done the day before hydrization step

Materials

One 650 μ l RNase-free tube labeled "P+" (Control for probe; not to be RNase treated)

One 650 μ l RNase-free tube labeled "P-" (Control: RNase-treated probe)

From Hybspeed RPA kit (Ambion):

Elution buffer from, ~130 μ l per sample to be run in Rnase Protection assay

Yeast RNA from Hybspeed RPA kit (Ambion), 10 μ l per sample

5M Ammonium Acetate from Hybspeed RPA kit, 0-13 μ l per sample

Combined radioactive probes from Section C, 25,000 cpm per probe per sample

One 1.0 μ g aliquot of each cellular RNA sample to be probed (each in 650 μ l RNase-free tubes).

Ideally, this should include an aliquot or aliquots of cellular RNA which are known from previous assays to contain the RNA targeted by the probes being used (positive control) and one aliquot of cellular RNA from fibroblasts known not to express chondrospecific genes (negative control).

Cold 100% Ethanol, 375 μ l per sample

Procedures

- 1) Combine $(n+2)10\ \mu\text{l}$ Yeast RNA + $(n+2)130\ \mu\text{l}$ Elution buffer in one tube where n = # of samples, including all controls, with volume of less than $10\ \mu\text{l}$
- 2) To above mix, add $(n+2)y\ \mu\text{l}$ of probe mix from section C
where n = # of samples, including all controls, with volume of less than $10\ \mu\text{l}$
where $y\ \mu\text{l}$ = volume of probe mix which contains 25,000 cpm of each probe
- 3) Add $(140 + y)\ \mu\text{l}$ to:
empty tube labeled P+
empty tube labeled P-
each tub with cellular RNA samples unless volume of RNA sample exceeds $10\ \mu\text{l}$
- 4) To each RNA sample with volume exceeding $10\ \mu\text{l}$, add:
 $1/10^{\text{th}}$ volume of 5M Ammonium Acetate
 $10\ \mu\text{l}$ of Yeast RNA
Enough Elution buffer to bring total volume to $140\ \mu\text{l}$
 $y\ \mu\text{l}$ (see above) of combined probe
- 5) Briefly vortex samples, add $375\ \mu\text{l}$ (~2.5 volumes) of cold 100% EtOH, and invert ~40X
- 6) Place all tubes and unused probe in "hot" freezer to allow co-precipitation for 1 hr or o/n

Day 2:

E. Hybridization of cellular RNA with antisense probes and Rnase treatment to remove ssRNA

See HybSpeed™ RPA (Ambion, Cat # 1412) Instruction Manual for additional information on background, kit components, additional procedures, and troubleshooting.

Materials

Three Heating blocks for $650\ \mu\text{l}$ tubes, set for 95° , 68° , & 37° (near radioactive shields)

From HybSpeed RPA kit (Ambion, Cat # 1412):

Rnase Digestion Buffer, $100\ \mu\text{l}$ per cellular RNA and probe control samples

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Hybridization Buffer, 10 µl per cellular RNA and probe control samples

Gel Loading Buffer II, 10 µl per cellular RNA and probe control samples, and for size marker.

One lane of size markers should be loaded per gel; one or two gels will be loaded, depending on number of samples (maximum of 10 lanes per gel)

Rnase A/T1 Mix (enzyme: keep in freezer until use and return to freezer immediately, 1 µl per sample

Inactive/Precipitation Mix (keep in freezer until use), 150 µl per cellular RNA and probe control samples

Co-precipitation tubes containing cellular RNA and probe stored o/n in freezer (Section D)

Microfuge in cold room

elongated pipetman tips (fine-tip)

vacuum flask designated for radioactive waste

radioactive shields (in Laboratory)

1% SDS, several mls for washing pipet tips

70% Ethanol, stored at -20°C, 300 µl per cellular RNA and probe control samples

Timer

Procedures

These Procedures may be performed in the lab (Radiation Room not necessary).

Perform procedures behind plexiglass radioactive shields, and use radioactive waste container.

Pay close attention to incubation times and temperatures.

- 1) Preheat one heating blocks and waterbath (These should all be in close proximity to each other)
- 2) Preheat Rnase digestion buffer to 37°C
- 3) Thaw Hybridization Buffer and Gel Loading Buffer II to room temperature
- 4) **Pellet RNA precipitate:** Remove o/n co-precipitation tubes from freezer (Section D) and microfuge at full speed for 15 minutes in cold room. Orient tubes such that you will know where the pellet is.

- 5) Using elongated pipetman tips (fine-tip), carefully aspirate supernatant (ethanol) into flask designated for radioactive waste.

Do not touch pellet. Rinse pipetman tip in 1% SDS between each tube.

- 6) Wash RNA pellet: To each pellet, gently (so as to not dislodge the pellet) add 300 μ l cold 70% Ethanol. Invert tubes gently ~4 times, and microfuge at full speed for 5 minutes in cold room.
- 7) During centrifugation, aliquot the amount of Hybridization Buffer needed and heat to 95°C
- 8) Repeat aspiration, removing as much Ethanol as possible from sides of tube without disturbing pellet.
- 9) Place pellets in 95°C heat block and add 10 μ l of preheated hybridization buffer to each tube.
- 10) Solubilization: Vortex each sample for a full 20 seconds; return each sample to 95°C immediately after vortexing
(Proceed quickly, vortexing two tubes at a time).
Revortex ~10 seconds each, returning each sample to 95°C immediately.
- "Resolubilization of the coprecipitated probe + RNA is essential for maximizing the sensitivity of the HybSpeed System. Do not be concerned by foaming that may occur."**
- 11) Hybridization: After 2-3 minutes at 95°C, transfer tubes quickly to 68°C waterbath and incubate for 10 minutes.
"Do not allow temperature of samples to drop"
- 12) During 10 minute hybridization, aliquot remove Rnase A/TI, vortex and microfuge briefly, and dilute 100:1 by adding 1 μ l into every 100 μ l of Rnase Digestion Buffer (buffer prewarmed to 37°C). Return stock Rnase to freezer. Briefly vortex and microfuge diluted Rnase. Keep diluted Rnase and unused Digestion Buffer at 37°C. **"Do not put on ice"**. You will need 100 μ l Digestion Buffer without Rnase for "P+" sample).
- 13) Digestion of non-hybridized ssRNA: At the end of the 10 minute hybridization, One tube at a time, transfer sample from 68°C bath directly to 37°C block and immediately add 100 μ l of diluted Rnase prewarmed to 37°C.

Exception: To tube labeled "P+" (see Section D), do not add diluted Rnase. Instead, add 100 µl of Digestion Buffer without Rnase. This sample will show the migration of intact probes during electrophoresis.

14) After all tubes have been treated as above, vortex each tube briefly and return to 37°C for 30 minutes, revortexing after the first 15 minutes. During this incubation, you may want to pour gel for electrophoresis (see below).

15) At end of 30' Rnase- treatment, add 150 µl of cold Inactivation/Precipitation Mix to each tube.

Vortex and microfuge briefly. Transfer tubes to -20°C freezer for at least 15 minutes (Can leave for several hours).

F. Electrophoresis of protected RNA

Pour 1 gel per maximum of 9 samples (not including marker lane)

Materials, per 4% acrylamide/8.3M Urea gel

From PAGE 1 Sequencing Gel Kit (Boehringer Cat# 100688):

Component 1: Acrylamide:Bisacrylamide (19:1)/8.3M Urea, 1.6 ml

Component 2: Diluent, 8.3 M Urea, 7.4 ml

10X TBE/8.3 M Urea (Boehringer Cat# 100919), 1.0 ml

Ammonium Persulfate (APS), 10% (prepared within one day) 70 µl

TEMED 10 µl

Running buffer: 1X TBE, ~300 ml

Gel pouring apparatus

Comb (10 teeth, 0.75 mm thick)

Gel electrophoresis apparatus

Fine tip pipetman tips

Electrophoresis power supply

Plastic wrap

BIOMAX MR Kodak film

Radioactive RNA size markers (transcribed as described in section B)

Samples in Inactivation/Precipitation Mix

Elongated pipetman tips (fine-tip)

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Vacuum flask designated for radioactive waste

Gel Loading Buffer II from HybSpeed RPA kit, 10 μ l per sample

Heating block for 650 μ l tubes set at 90°C

Procedures

- 1) Pour 4% acrylamide/8.3M Urea gel: follow steps 1-5 of Section C
- 2) Thaw radioactive size markers and Gel Loading Buffer
- 3) Remove samples in Inactivation/Precipitation Mix from freezer and microfuge 15 minutes at maximum speed in cold room
- 4) During centrifugation, remove plate-gel-backing unit from apparatus and rinse plates with tap distilled water. Remove comb and rinse well 2-3 times, shaking out water between rinses
- 5) Install plate-gel-backing in electrophoresis apparatus
- 6) Add 1X TBE to top reservoir checking for leaking, and then to bottom reservoir
- 7) After 15 minute centrifugation, using elongated pipetman tips (fine-tip), carefully aspirate supernatant (ethanol) into vacuum flask designated for radioactive waste.

Do not touch pellet. Rinse Pipetman tip in 1% SDS between each tube.

- 8) Microfuge 30 seconds full speed in cold room and aspirate again

"Residual supernatant will cause aberrant migration of bands in gel"

- 9) Add 10 μ l Gel Loading Buffer, vortex vigorously and microfuge briefly
- 10) Heat samples in heating block to 90°C for 3-4 minutes. Note: heating too long may cause samples to become trapped in well of gel.
- 11) Just before adding samples, rinse wells with buffer in top reservoir, using P1000
- 12) With samples in 90°C heating block, load 8 μ l of each sample per wells

Exceptions:

Markers: Dilute 1 μ l into 9 μ l Gel Loading Buffer and load only 1 μ l into well.

Markers do not need to be heated. As marker decays (half life = 2 weeks), increase amount of marker loaded accordingly.

"P+" control probe sample: load only 1 μ l

Note on loading: If possible, avoid use of end lanes and leave an empty well between the Marker lane and the adjacent sample

- 13) Hook up power supply and run gel at 125 V until fast dye is near end of gel
- 14) Remove plate/gel/backing from electrophoresis apparatus and rinse all components thoroughly with tap water.
- 15) Remove glass plate
- 16) Transfer gel from backing plate to Whatman paper and dry on gel dryer

G. Phosphoimager

- 1) Expose gel in erased phosphoimager cassette, recording position of gel on grid
- 2) After 1-4 days exposure, scan image and quantify bands

DNA fragments containing partial sequences of aggrecan (Agg) (Doege et al., J. Biol. Chem 266:894-902, 1991) and types I and II collagens (Kuivaniemi et al., Biochem J. 252:633-640, 1988 and Baldwin et al., Biochem J. 262:521-528, 1989) were generated by PCR amplification of human chondrocyte cDNA libraries. Paired oligonucleotides, representing coding sequences within each gene separated by several hundred basepairs (bp) were used as primers for PCR. Included at the 5' end of the downstream primer, was an anchor sequence (CAGTGCCAT) for subsequent addition of the T7 RNA polymerase promoter. The sequence of the primers with upstream and downstream sequences shown respectively in 5' to 3' orientation are as follows:

- i) CCATGCAATTTGAGAACT (SEQ ID No:1); and
- ii) ACAAGAAGAGGACACCGT (SEQ ID No:2) to generate 551 bp of aggrecan gene sequence (Agg₅₅₁);
- iii) CCATGCAATTTGAGAACT (SEQ ID No:3); and
- iv) CTTGATGGTCCTGTCTGTTTCAG (SEQ ID No:4); for Agg₃₀₇;
- v) GCGGAATTCCCCCAGCCACAAAGAGTC (SEQ ID No:5); and
- vi) CGTCATCGCACAAACACCT (SEQ ID No:6) for 261 bp of the type II collagen (CI) gene; and
- vii) GTCCCCGTGGCCTCCCCG (SEQ ID No:7); and
- viii) CCACGAGCACCAGCACTT (SEQ ID No:8) for 307 bp of type II collagen gene (CII).

The amplified fragments were inserted into pCRscript vector (Stratagene, LaJolla California) for the propagation and maintenance. In order to generate templates for

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the transcription of antisense probes, a second PCR amplification was performed using these cloned cDNA fragments. For priming, respective upstream primers shown above were each paired with the T7 promoter sequence containing the same anchor sequence (underlined) that use used in the first PCR amplification:

GGAATTCTTAGATAATACGACTCACTATAGGGCAGTGCCAT (SEQ ID No:9);. DNA templates containing either 80 bp of the 18S rRNA gene or 316 bp of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, each linked to an upstream T7 promoter, were supplied by Ambion (Austin, Texas). Prior to use as a template, GAPDH sequence linked to the promoter was reduced to 149 bp by digestion with Dde I.

In vitro transcription from the above templates was performed using the Maxiscript™ kit (Ambion) according to manufacturer's instructions. Full-length probes were purified from the transcription reaction by electrophoresis on 7M urea, 4% polyacrylamide 1X TBE gels, followed by autoradiography, excision from the gel of bands corresponding to the full length transcripts, and passive diffusion into probe elution buffer (supplied in the Maxiscript™ kit) for two hours at 37°C. The activity of the probe was quantified by scintillation counting.

RNase protection assays were performed using the Hybspeed™ RPA kit (Ambion) according to manufacturer's instructions. Briefly, radiolabelled antisense RNA probes for aggrecan and types I and II collagens were combined and hybridized with RNA isolated from chondrocytes, using an excess of probe. A probe for 18S rRNA or GAPDH was also included in each hybridization mixture to normalize for total RNA. For negative controls, yeast RNA alone was combined with probes. For positive controls, probes were hybridized to RNA samples know to contain sequences complementary to all four probes. Digestion with an RNaseA/RNase T1 mix was performed to degrade unhybridized RNAs. Hybridized RNAs protected from digestion were resolved by electrophoresis as described above and visualized by autoradiography or by using a Fujifilm BAS-1500 phosphorimager. Bands on the phoshorimage representing types I and II collagen genes were quantified using MacBAS version 2.4 software. Any signal from the corresponding position of the negative control (no chondrocyte RNA) was subtracted.

Cells in alginate culture were grown in the basal medium described above, supplemented as follows:

Culture 1: 1X ITS+

Culture 2: 1XITS+ and 0.2 ng/ml TGF- β 1

Culture 3: 1XITS+ and 1.0 ng/ml TGF- β 1

Culture 4: 1XITS+ and 5.0 ng/ml TGF- β 1

Cells were harvested at 7 and 21 days for RNA isolation.

The results of Rnase Protection Assay on RNA from the 7-day cultures showed that in the absence of TGF- β 1 (culture 1), there was little or no detectable CII or Agg mRNA while CI mRNA was abundant. With addition of 0.2 ng/ml TGF- β 1 (culture 2) there was a clear induction of mRNA abundance for the chondrocyte differentiation markers CII and Agg, while CI abundance was not significantly altered. Addition of higher TGF- β 1 concentrations (cultures 3 and 4) showed a dose-dependent increase CII and Agg with no change in CI. Cultures harvested at 21 days yielded similar results. In a separate example we showed that a 100-fold molar excess of a monoclonal neutralizing antibody against TGF- β , when included with the culture supplements listed for culture 3, yielded results similar to that of culture 1. This effectively eliminates the possibility that the differentiating activity was due to a contaminant of the TGF- β 1 preparation.

Example 8

Culture conditions for cultures 1, 3 and 4 of example 7 were repeated. In parallel cultures, TGF- β 2 was used in place of TGF- β 1. The results from the TGF- β 1 and β 2 cultures were similar to the corresponding cultures from Example 7, indicating that TGF- β 1 and β 2 have similar properties with respect to induction of chondrogenesis in this culture system.

Example 9

Chondrocytes embedded in alginate were cultured in basal medium supplemented with ITS+ and 1 ng/ml TGF- β 2 for 1,2,4,7, and 21 days. As a negative

control, cells were cultured for 21 days in basal medium supplemented with IGS+ alone. RNA analysis of these cultures showed a general trend of increasing CII and

Agg RNA throughout the first seven days (~5-fold increase in aggrecan and ~40-fold increase in CII). At day 21, the abundance of CII and Agg mRNA apparently dropped off, but remained high compared to day 1.

Example 10

As shown in appendix C, 1XITS+ is a mixture of several components including 6.25 µg/ml insulin. This example was performed to determine whether, in the above examples, the insulin in ITS+ was playing a role in TGF-β mediated induction of CII and Agg. Secondly, if insulin was playing a role, we wanted to see if it can be replaced by IGF-I. The culture condition of culture 3 in example 7 was repeated. In parallel cultures, ITS+ media was reproduced with insulin omitted or replaced with 10 ng/ml IGF-I. The results from 7 day cultures showed that in the absence of insulin and IGF-I, 1 ng/ml TGF-β induced neither CII nor Agg expression. However, addition to the culture of 10 ng/ml IGF-I in lieu of 6.25 µg/ml insulin, restored TGF-β1 mediated induction of these chondrogenic markers to levels comparable to that of condition 3 of example 7. This suggests that the IGF receptor, which binds insulin with low affinity (Schmid, 1995), needs to be activated in order for TGF-β mediated chondrogenesis to occur. Furthermore, the use of IGF-I at approximately 600-fold lower concentration than that of insulin substantially reduces the possibility of contaminating factors affecting differentiation.

In a separate example, other components of the ITS+ were omitted or substituted. We found that transferrin and selenious acid could be removed without consequence, and that human serum albumin can replace bovine serum albumin.

In conclusion, a complete defined medium, that includes basal medium supplemented with 1 ng/ml TGF-β1 or β2, 10 ng/ml IGF-I, 1 mg/ml human serum albumin, and may further include 5 µg/ml linoleic acid, will induce de-differentiated human chondrocytes to re-express the chondrocyte differentiated markers CII and Agg in suspension cultures.

THE INVENTION CLAIMED IS:

1. A defined cell culture medium, comprising:
 - (a) a supplement mixture comprising ITS, hydrocortisone, FGF, fibronectin, and IGF;
 - (b) a component mixture comprising: glucose, glutathione, hypoxanthine, linoleic acid, lipoic acid, putrescine, sodium pyruvate, and thymidine;
 - (c) a vitamin mixture, comprising: niacinamide, inositol, folic acid, choline chloride, calcium pantothenate, pyridoxal, riboflavin, vitamin B₁₂, pyridoxine, biotin, para-aminobenzoic acid, and thiamine;
 - (d) an inorganic salt mixture, comprising: CaCl₂, Ca(NO₃)₂, CuSO₄, Fe(NO₃)₃, FeSO₄, KCl, MgSO₄, MgCl₂, NaCl, NaHCO₃, NaH₂PO₄, Na₂HPO₄, and ZnSO₄; and
 - (e) an amino acid mixture, comprising: alanine, arginine, asparagine, aspartic acid, cystine, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.
2. The defined cell culture medium of claim 1, wherein the supplement mixture comprises, per liter of medium: about 1% of ITS, about 40,000ng of hydrocortisone, about 1,000ng of FGF, about 5,000µg of fibronectin, and about 1,000 ng of IGF.
3. The defined cell culture medium of claim 1, wherein the component mixture comprises, per liter of medium: about 8,302 mg of glucose, about 1 mg of glutathione, about 4.77 mg of hypoxanthine, about 0.084 mg of linoleic acid, about 0.21 mg of lipoic acid, about 0.161 of mg of putrescine, about 110 mg of sodium pyruvate, and about 0.70 mg of thymidine.

4. The defined cell culture medium of claim 1, wherein the vitamin mixture comprises, per liter of medium: about 4.75 mg of calcium pantothenate, about 21 mg of choline chloride, about 6.30 mg of folic acid, about 60.20 mg of inositol, about 5.036 mg of niacinamide, about 4 mg of pyridoxal, about 0.637 mg of riboflavin, about 5.30 mg of thiamine, about 1.405 mg of Vitamin B₁₂, about 1.06 mg of pyridoxine, about 1 mg of para-aminobenzoic acid, and about .2073 mg of biotin.
5. The defined cell culture medium of claim 1, wherein the inorganic salt mixture comprises, per liter of medium: about 233.22 mg of CaCl₂, about 100 mg of Ca(NO₃)₂, about 0.0024 mg of CuSO₄, about 0.10 mg of Fe(NO₃)₃, about 0.83 mg of FeSO₄, about 1023.60 mg of KCl, about 146.51 mg of MgSO₄, about 57.22 mg of MgCl₂, about 19999 mg of NaCl, about 6876 mg of NaHCO₃, about 125 mg of NaH₂PO₄, about 942 mg of Na₂HPO₄, and about 0.86 mg of ZnSO₄.
6. The defined cell culture medium of claim 1, wherein the amino acid mixture comprises, per liter of medium: about 8.9 mg of alanine, about 495 mg of arginine, about 65.01 mg of asparagine, about 33.30 mg of aspartic acid, about 128 mg of cystine, about 35.12 mg of cysteine, about 34.70 mg of glutamic acid, about 1030 mg of glutamine, about 47.50 mg of glycine, about 78 mg of histidine, about 20 mg of hydroxyproline, about 159 mg of isoleucine, about 168.10 mg of leucine, about 222.50 mg of lysine, about 49.50 mg of methionine, about 86 mg of phenylalanine, about 54.50 mg of proline, about 82.50 mg of serine, about 126.90 mg of threonine, about 23 mg of tryptophan, about 140.81 mg of tyrosine, and about 125.70 mg of valine.
7. A defined cell culture medium, comprising, per liter of medium:
 - (a) a supplement mixture comprising: about 1% of ITS, about 40,000ng of hydrocortisone, about 1,000ng of FGF, about 5,000µg of fibronectin, and about 1,000 ng of IGF;

- (b) a component mixture comprising: about 8,302 mg of glucose, about 1 mg of glutathione, about 4.77 mg of hypoxanthine, about 0.084 mg of linoleic acid, about 0.21 mg of lipoic acid, about 0.161 mg of putrescine, about 110 mg of sodium pyruvate, and about 0.70 mg of thymidine;
- (c) a vitamin mixture, comprising: about 4.75 mg of calcium pantothenate, about 21 mg of choline chloride, about 6.30 mg of folic acid, about 60.20 mg of inositol, about 5.036 mg of niacinamide, about 4 mg of pyridoxal, about 0.637 mg of riboflavin, about 5.30 mg of thiamine, about 1.405 mg of Vitamin B₁₂, about 1.06 mg of pyridoxine, about 1 mg of para-aminobenzoic acid, and about .2073 mg of biotin;
- (d) an inorganic salt mixture, comprising: about 233.22 mg of CaCl₂, about 100 mg of Ca(NO₃)₂, about 0.0024 mg of CuSO₄, about 0.10 mg of Fe(NO₃)₃, about 0.83 mg of FeSO₄, about 1023.60 mg of KCl, about 146.51 mg of MgSO₄, about 57.22 mg of MgCl₂, about 19999 mg of NaCl, about 6876 mg of NaHCO₃, about 125 mg of NaH₂PO₄, about 942 mg of Na₂HPO₄, and about 0.86 mg of ZnSO₄; and
- (e) an amino acid mixture, comprising: about 8.9 mg of alanine, about 495 mg of arginine, about 65.01 mg of asparagine, about 33.30 mg of aspartic acid, about 128 mg of cystine, about 35.12 mg of cysteine, about 34.70 mg of glutamic acid, about 1030 mg of glutamine, about 47.50 mg of glycine, about 78 mg of histidine, about 20 mg of hydroxyproline, about 159 mg of isoleucine, about 168.10 mg of leucine, about 222.50 mg of lysine, about 49.50 mg of methionine, about 86 mg of phenylalanine, about 54.50 mg of proline, about 82.50 mg of serine, about 126.90 mg of threonine, about 23 mg of tryptophan, about 140.81 mg of tyrosine, and about 125.70 mg of valine.

8. A method of culturing eukaryotic cells, comprising the step of adding to eukaryotic cells a defined medium comprising:

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- (a) a supplement mixture comprising: ITS, hydrocortisone, FGF, fibronectin, and IGF;
- (b) a component mixture comprising: glucose, glutathione, hypoxanthine, linoleic acid, lipoic acid, putrescine, sodium pyruvate, and thymidine;
- (c) a vitamin mixture, comprising: niacinamide, inositol, folic acid, choline chloride, calcium pantothenate, pyridoxal, riboflavin, biotin, para-aminobenzoic acid, pyridoxine, vitamin B₁₂, and thiamine;
- (d) an inorganic salt mixture, comprising: CaCl₂, Ca(NO₃)₂, CuSO₄, Fe(NO₃)₃, FeSO₄, KCl, MgSO₄, MgCl₂, NaCl, NaHCO₃, NaH₂PO₄, Na₂HPO₄, and ZnSO₄; and
- (e) an amino acid mixture, comprising: alanine, arginine, asparagine, aspartic acid, cystine, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine,

wherein the defined medium maintains the viability of the eukaryotic cells.

- 9. The method of claim 8, wherein the supplement mixture comprises, per liter of medium: about 1% of ITS, about 40,000ng of hydrocortisone, about 1,000ng of FGF, about 5,000µg of fibronectin, and about 1,000 ng of IGF.
- 10. The method of claim 8, wherein the component mixture comprises, per liter of medium: about 8,302 mg of glucose about 1 mg of glutathione, about 4.77 mg of hypoxanthine, about 0.084 mg of linoleic acid, about 0.21 mg of lipoic acid, about 0.161 of mg of putrescine, about 110 mg of sodium pyruvate, and about 0.70 mg of thymidine.
- 11. The method of claim 8, wherein the vitamin mixture comprises, per liter of medium: about 4.75 mg of calcium pantothenate, about 21 mg of choline

chloride, about 6.30 mg of folic acid, about 60.20 mg of inositol, about 5.036 mg of niacinamide, about 4 mg of pyridoxal, about 0.637 mg of riboflavin, about 5.30 mg of thiamine, about 1.405 mg of Vitamin B₁₂, about 1.06 mg of pyridoxine, about 1 mg of para-aminobenzoic acid, and about .2073 mg of biotin.

12. The method of claim 8, wherein the inorganic salt mixture comprises, per liter of medium: about 233.22 mg of CaCl₂, about 100 mg of Ca(NO₃)₂, about 0.0024 mg of CuSO₄, about 0.10 mg of Fe(NO₃)₃, about 0.83 mg of FeSO₄, about 1023.60 mg of KCl, about 146.51 mg of MgSO₄, about 57.22 mg of MgCl₂, about 19999 mg of NaCl, about 6876 mg of NaHCO₃, about 125 mg of NaH₂PO₄, about 942 mg of Na₂HPO₄, and about 0.86 mg of ZnSO₄.
13. The method of claim 8, wherein the amino acid mixture comprises, per liter of medium: about 8.9 mg of alanine, about 495 mg of arginine, about 65.01 mg of asparagine, about 33.30 mg of aspartic acid, about 128 mg of cystine, about 35.12 mg of cysteine, about 34.70 mg of glutamic acid, about 1030 mg of glutamine, about 47.50 mg of glycine, about 78 mg of histidine, about 20 mg of hydroxyproline, about 159 mg of isoleucine, about 168.10 mg of leucine, about 222.50 mg of lysine, about 49.50 mg of methionine, about 86 mg of phenylalanine, about 54.50 mg of proline, about 82.50 mg of serine, about 126.90 mg of threonine, about 23 mg of tryptophan, about 140.81 mg of tyrosine, and about 125.70 mg of valine.
14. A method of culturing an eukaryotic cell line, comprising the step of culturing the eukaryotic cell line in a defined cell culture medium, comprising, per liter of medium:
 - (a) a supplement mixture comprising: about 40,000ng of hydrocortisone, about 1,000ng of FGF, about 5,000μg of fibronectin, and about 1,000 ng of IGF;
 - (b) a component mixture comprising: about 8302 mg of glucose, about 1 mg of glutathione, about 4.77 mg of hypoxanthine, about 0.084

mg of linoleic acid, about 0.21 mg of lipoic acid, about 0.161 mg of putrescine, about 110.00 mg of sodium pyruvate, and 0.70 mg of thymidine;

- (c) a vitamin mixture, comprising: about 4.75 mg of calcium pantothenate, about 21 mg of choline chloride, about 6.30 mg of folic acid, about 60.20 mg of inositol, about 5.036 mg of niacinamide, about 4 mg of pyridoxal, about 0.637 mg of riboflavin, about 5.30 mg of thiamine, about 1.405 mg of Vitamin B₁₂, about 1.06 mg of pyridoxine, about 1 mg of para-aminobenzoic acid, and about .2073 mg of biotin;
- (d) an inorganic salt mixture, comprising: about 233.22 mg of CaCl₂, about 100 mg of Ca(NO₃)₂, about 0.0024 mg of CuSO₄, about 0.10 mg of Fe(NO₃)₃, about 0.83 mg of FeSO₄, about 1023.60 mg of KCl, about 146.51 mg of MgSO₄, about 57.22 mg of MgCl₂, about 19999 mg of NaCl, about 6876 mg of NaHCO₃, about 125 mg of NaH₂PO₄, about 942 mg of Na₂HPO₄, and about 0.86 mg of ZnSO₄; and
- (e) an amino acid mixture, comprising: about 8.9 mg of alanine, about 495 mg of arginine, about 65.01 mg of asparagine, about 33.30 mg of aspartic acid, about 128 mg of cystine, about 35.12 mg of cysteine, about 34.70 mg of glutamic acid, about 1030 mg of glutamine, about 47.50 mg of glycine, about 78 mg of histidine, about 20 mg of hydroxyproline, about 159 mg of isoleucine, about 168.10 mg of leucine, about 222.50 mg of lysine, about 49.50 mg of methionine, about 86 mg of phenylalanine, about 54.50 mg of proline, about 82.50 mg of serine, about 126.90 mg of threonine, about 23 mg of tryptophan, about 140.81 mg of tyrosine, and about 125.70 mg of valine,

wherein the defined medium maintains the viability of the eukaryotic cells.

15. A method of enhancing the differentiation of fibroblasts by using a composition containing TGF- β .

16. The method of claim 15, wherein the fibroblasts are chondrocytes.
17. The method of claim 15, wherein the fibroblasts are dedifferentiated.
18. A method of enhancing the synthesis of a cartilage specific matrix by fibroblasts using TGF- β .
19. A method of enhancing the differentiation of chondrocytes using a composition containing TGF- β and IGF.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/00, 5/08		A3	(11) International Publication Number: WO 98/04681
			(43) International Publication Date: 5 February 1998 (05.02.98)
(21) International Application Number: PCT/US97/13140		YAEGER, Peter, C. [US/US]; 19 Robin Hood Road, Natick, MA 01760 (US). BROWN, Marie, E. [NL/US]; 21 Mosman Street, West Newton, MA 02165 (US). HANLON, James, G. [US/US]; 105 High Street, Ashland, MA 01721 (US). BINETTE, Francois [CA/US]; 40 Crescent, Belmont, MA 02178 (US). (74) Agents: GOSZ, William, G. et al.; Genzyme Corporation, One Mountain Road, Framingham, MA 01701 (US). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 18 June 1998 (18.06.98)	
(22) International Filing Date: 25 July 1997 (25.07.97)			
(30) Priority Data: 60/022,801 25 July 1996 (25.07.96) US 60/022,810 25 July 1996 (25.07.96) US 60/022,711 26 July 1996 (26.07.96) US			
(60) Parent Applications or Grants (63) Related by Continuation US 60/022,801 (CIP) Filed on 25 July 1996 (25.07.96) US 60/022,810 (CIP) Filed on 25 July 1996 (25.07.96) US 60/022,711 (CIP) Filed on 26 July 1996 (26.07.96)			
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(72) Inventors; and (75) Inventors/Applicants (for US only): MCPHERSON, John, M. [US/US]; 378 Elm Street, Hopkinton, MA 01748 (US).			
(54) Title: CHONDROCYTE MEDIA FORMULATIONS AND CULTURE PROCEDURES			
(57) Abstract <p>One object of the present invention is based upon the development and use of a serum-free defined cell culture medium comprising a supplement mixture, a component mixture, a vitamin mixture, an inorganic salt mixture and amino acid mixture that avoids the problems inherent in the use of serum. In particular, the defined medium is useful in culturing fibroblasts, especially chondrocytes. Another object of the present invention is to claim a method of enhancing the differentiation of chondrocytes and enhancing the synthesis of a cartilage specific matrix using tumor growth factor beta (TGF-β). Another object of the present invention is to claim a method of enhancing the differentiation of chondrocytes using the combination of TGF-β and IGF.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/13140

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADOLPHE ET AL: "CELL MULTIPLICATION AND TYPE II COLLAGEN PRODUCTION BY RABBIT ARTICULAR CHONDROCYTES CULTIVATED IN A DEFINED MEDIUM" EXPERIMENTAL CELL RESEARCH, vol. 155, 1984, pages 527-536, XP002046800 cited in the application see the whole document	1-14
A	US 5 328 844 A (MOORE GEORGE E) 12 July 1994 see column 1, line 8 - line 10 see column 3, line 5 - line 17	1-14
A	WO 95 00632 A (AMGEN INC) 5 January 1995 see page 7, line 33 - page 10, line 8	1-14
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

13 November 1997

Date of mailing of the international search report

29. 04. 98

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13140

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 343 635 A (TEIJIN LTD) 29 November 1989 see page 23, line 43 - line 48 ---	1-14
A	EP 0 295 605 A (TOSOH CORP) 21 December 1988 see column 3, line 31 - column 4, line 1 ---	1-14
A	WO 96 12793 A (AGRONOMIQUE INST NAT RECH ;CENTRE NAT RECH SCIENT (FR); ECOLE NORM) 2 May 1996 see page 3, line 17 - page 6, line 15 -----	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/13140

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
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Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/13140

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-14

A defined cell culture medium comprising a supplement mixture, a component mixture, a vitamin mixture, an inorganic salt mixture and an amino acid mixture, method of culturing eukaryotic cells using such a defined medium.

2. Claims: 15-19

A method of enhancing the differentiation of fibroblasts using TGF-Beta, a method of enhancing the synthesis of a cartilage specific matrix by fibroblasts using TGF-Beta

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/13140

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